Protective effects of hydroxysafflor yellow an on high oxidized low density lipoprotein induced human coronary artery endothelial cells injuries

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Abstract: The aim of this study was to investigate the injury of human coronary artery endothelial cells (HCAECs) induced by high oxidized low density lipoprotein (ox-LDL) and to examine the protective effect of hydroxysafflor yellow A (HSYA) on HCAECs injury. It was found that in the high ox-LDL group the content of NO, the expression of endothelial nitric oxide synthase (eNOS) mRNA and protein were decreased. The expression of LDL, lectin-like low-density lipoprotein receptor 1 (LOX-1) mRNA and LOX-1 protein were up-regulated. The number of apoptotic cells were significantly increased after dealt with high ox-LDL compared with the control group. Furthermore, in the high ox-LDL+HSYA group, the cell survival rate, the release of NO and the expression of eNOS mRNA and eNOS protein were increased. The secretion of LDL and the expression of LOX-1 at the level of mRNA and protein were down-regulated. The number of apoptosis were significantly reduced after combined with HSYA compared with the high ox-LDL group. Therefore, high ox-LDL had an obvious damage to HCAECs. The HSYA inhibited the high ox-LDL-induced HCAECs injury and protected and promoted the cell repaired, possibly by up-regulating the eNOS gene and protein expression, increasing NO release, inhibiting LDH release and down-regulating LOX-1 mRNA and protein expression to achieve the effect. The treatment and secondary prevention of coronary heart disease, as well as the recovery of patients undergoing postoperative percutaneous coronary intervention (PCI), will benefit to a large extent.

Keywords: Hydroxysafflor yellow A; High oxidized low density lipoprotein; Lectin-like low-density lipoprotein receptor 1; Human coronary endothelial cells; Atherosclerosis; Cell apoptosis

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1. Introduction

HSYA is one of the main biologically active and water-soluble compounds which was isolated from Carthamus tinctorius L. HSYA was first isolated by Meselhy et al. in 1993[1], which was the main active ingredient of Carthamus tinctorius L. Its structure had been confirmed to be C-glycosylquinolone[2]. In 2005, HSYA-containing safflower injections were officially approved by the State Food and Drug Administration of China as new clinical cardiovascular drugs, and began to be widely used to treat cardiovascular diseases such as angina[3]. The therapeutic effect of HSYA on cardiovascular disease was related to its anticoagulant effect, anti-myocardial ischemic activity and vasodilation[4]. However, in the process of atherosclerosis, how HSYA affects the development of coronary heart disease by acting on coronary endothelial cells is still not clear.

Studies have shown that ox-LDL induced the endothelial dysfunction and apoptosis play a key role in the pathogenesis of atherosclerosis[5-7]. LOX-1 is an important multifunctional receptor that affects cardiovascular function[3]. It was first identified as a receptor of ox-LDL in endothelial cells[8]. Previous studies have found that ox-LDL is the strongest activator of LOX-1. Activation of LOX-1 can cause endothelial cells to inflammatory, promote apoptosis and cause endothelial cell dysfunction, and participate in the pathogenesis of atherosclerosis[7, 9-12]. At the same time, researches found that inhibition of LOX-1 can greatly help alleviate cardiovascular diseases[13-15]. In our study, we simulated the processes of atherosclerosis in humans by inducing functional impairment of HCAECs with high ox-LDL in vitro. Furthermore, we examined the protective effect of HSYA on high ox-LDL-induced damage to HCAECs.

Coronary artery disease is a disease in which vascular stenosis is caused by atherosclerotic plaque obstruction of the coronary arteries[16]. A structurally complex atherosclerotic plaque plays a major role in
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the development of coronary heart disease (CHD)[17]. Among them, endothelial dysfunction, lipid aggregation, immune cell infiltration, hyperplastic connective tissue and atherosclerotic plaque formation are considered to be the main features and manifestations of atherosclerosis[18]. It can lead to thickening of the arterial wall and loss of elasticity of the blood vessels, eventually leading to the occurrence of various severe arterial occlusive diseases[19,20]. But so far, the underlying pathogenesis of atherosclerosis is not fully understood. Therefore, how to protect endothelial cells from functional damage during atherosclerosis is the key to prevent atherosclerosis, and it is even possible to reverse the atherosclerotic processes.

2. Materials and Methods

2.1. Experimental grouping

HCAECs were divided into four groups: the control group (no special deal with the HCAECs), the HYSA group (deal with the HYSAs (0.2mM)), the high oxLDL group (deal with the High oxLDL (20ug/ul)), the HYSA+High oxLDL group (combine the HYSA (0.2mM) with the High oxLDL (20ug/ul)). The HYSA was purchased from Targetmol of America and the high oxLDL purchased from Yiyuan Biotechnology of China.

2.2. Cell culture and establishment of cell injury model

HCAECs were cultured in RPMI-1640 medium (USA, Lonza) supplemented with 10% fetal bovine serum (Australia, ExCell Bio) in a humidified atmosphere containing 5% CO2 at 37°C. Cells from the fifth to the fifteenth generation were used in this study. When the cell fusion density reached to 60-70%, the high ox-LDL (20ug/ul) was used to stimulate the HCAECs for 24 hours to set up the cell injury model. And the MTT (Methylthiazolylidiphenyl-tetrazolium bromide) was used to detect the damage efficiency.

2.3. Cell viability, NO, and LDH content determination

HCAECs were seeded into 96-well plates at a density of 1x 10^4 cells/well. When the cell fusion density reached 60-70%, cells were treated with HYSAs, high oxLDL, and HYSA + high oxLDL for 24 hours. Cell viability was assessed by MTT assay using a multi-function microplate reader (USA, Thermo Fisher Scientific) to measure absorbance at 490 nm according to the manufacturer's instructions (USA, Sigma). With the same pre-processing method, HCAECs were seeded into a 24-well plate at a density of 5x 10^4 cells/well, and the same drug treatment method treated for 24 hours. The total NO and LDH released into the medium were detected by using the nitrate acid assay kit (China, Beyotime) and the LDH assay kit (China, Nanjing Institute of Bioengineering) according to the manufacturer's instructions.

2.4. TUNEL for detection of apoptosis in HCAECs

Samely, HCAECs were cultured at a density of 10^5 cells/well in a 24-well plate pre-placed with appropriate pre-treated sterile coverslips per well. Cells treated as described above. The proportion of apoptotic cells was measured using the TUNEL kit (China, Yeasen Biotech Co., Ltd.) for apoptosis according to the manufacturer's instructions, and the apoptosis rate of the cells was analyzed.

2.5. Western-blot detection for LOX-1 and eNOS protein expression

HCAECs was seeded into 6-well plates at a density of 20 x 10^4 cells/well. The cells were treated in the same manner as described above. Total cellular protein was extracted by RIPA lysis (China, Suo Laibao Technology Co., Ltd.) and then transferred to a nitrocellulose membrane. After incubation for 1h in blocking solution (5% skim milk), the membrane was incubated with 1:1000 dilution of primary antibody to detect β-actin (USA, abcom), LOX-1 (USA, abcom) and eNOS (USA, CST), overnight at 4°C. Washed membranes were incubated with 1:3000 dilution of horseradish peroxidase (HRP)-labeled anti-mouse/anti-rabbit secondary antibody (USA, abcom) with 5% skim milk powder blocking buffer for 1 hour and detected using a Vilber Solo 4S chemiluminescence imaging system. The relative strength was analyzed by Image J software (USA, Media Cybernetics) analysis.

2.6. RT-qPCR for LOX-1 mRNA and eNOS mRNA expression

HCAECs was seeded into 6-well plates at a density of 20 × 10^4 cells/well, cell treatment as described above for 24 hours, and then using the Trizol method to extract total cellular RNA and using the Prime Script RT reagent Kit (Japan, TakaRa) and to reverse RNA to cDNA according to the manufacturer's instructions. Also according to the protocol, SYBR Premix Ex Taq II (Japan, TakaRa) chimeric fluorescence for Real Time PCR. Primer synthesis from a biological company (China, Qingdao Yingke Yuki Bio), the primer base sequence was as follows: LOX-1 Forward primer: 5'-GAGAGTAGCAAATTGGTCCTCCTT-3'; Reverse primer: 5'-GCCCGAGGAAAATAGGTAACAGT-3'; e NOS Forward primer: 5'-TCTCCGCTCAGCTCAT-3';
Reverse primer: 5'-AGCCA TACAGGA TTGTCGCC-3',
GAPDH Forward primer: 5'-AGGTCGGTGTAACGGATTTG-3',
Reverse primer: 5'-TGTAAGCATTAGTTGAGGTC-3'. The RT-PCR reaction conditions were as follows: activation at 95°C for 30s, denaturation and extension at 95°C for 5s, 59°C for 30s, for a total of 40 cycles.

2.7. Statistics
Statistical analysis using Student's t-test was completed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Differences described as p ≤ 0.05 were considered significant. The results were presented as the mean ± standard deviation (SD).

Figure 1. Effect of HYSA and high ox-LDL on HCAEC cell viability (%). A. Compared with the control group, the difference in cell viability between different HSYA concentration groups is statistically significant. The HSYA 0.2mM group was significantly higher than the HSYA 0.4mM group, the HSYA 0.8mM group and the HSYA 1.6mM group. The difference between the HSYA 0.4mM group and HSYA 0.8mM group were not statistically significant (n=9). B. Compared with the control group, the 24h survival rate of the different High ox-LDL concentration groups was statistically significant. The high ox-LDL 5.0 μg/ml group > high ox-LDL 10.0 μg/ml group > high ox-LDL 20.0 μg/ml group > high ox-LDL 50.0 μg/ml group, The differences between the groups were statistically significant (n=14). C. There was a statistically significant difference in cell viability between different groups. The control group> high ox-LDL (20μg/ml) group> high ox-LDL (20μg/ml)+HSYA (0.2mM) group> HSYA (0.2mM) group, the difference between the two groups was statistically significant (n=15). The values (mean±SD from three independent experiments) are relative to control, which was set as **P<0.01 vs. control.
Figure 2. Effects of high ox-LDL and HSYA on the expression levels of NO and LDH in cells. A. The NO content (μM) showed high ox-LDL group < control group, high ox-LDL group < high ox-LDL + HSYA group, the difference between the two groups was statistically significant (n=9). B. The LDH content (U/dL) was expressed in the control group < HSYA group < high ox-LDL + HSYA group < high ox-LDL group. The difference between the two groups was statistically significant. The values (mean±SD from three independent experiments) are relative to control, which was set as **P<0.01 vs. control (n=9).

Figure 3. Effects of HYSA on high oxLDL-induced HCAECs apoptosis. A: TUNEL staining images; B: Rate of apoptotic cells positive staining TUNEL cell. The apoptosis rate were: control group 2.02% < HSYA group 4.6% < high ox-LDL + HSYA group 31.88% < high ox-LDL group 46.16%. The values (mean±SD from three independent experiments) are relative to control, which was set as **P<0.01 vs. control.
Figure 4. Effects of HYSA on high oxLDL-induced alteration of LOX-1, eNOS protein expression. A: LOX-1 and eNOS protein expressions analyzed by Western-blot; B/C: Relative quantitative analysis of LOX-1 and eNOS protein expression. The values (mean±SD from three independent experiments) are relative to control, which was set as *P<0.05, **P<0.01 vs. control.

Figure 5. Effects of HYSA on high oxLDL-induced alteration of LOX-1, eNOS mRNA expression. A/B: LOX-1 and eNOS mRNA expressions were analyzed by quantitative real-time PCR. The values (mean±SD from three independent experiments) are relative to control, which was set as *P<0.05, **P<0.01 vs. control.
3. Results

3.1. Effects of HYSA on high oxLDL-induced cell viability alteration

With the MTT assay, the 24-hour survival rate of cells were evaluated to determine the optimal working concentration of HYSA (Figure 1A). Cells treated with different concentrations of HYSA showed statistically significant differences in cell viability compared with the control group. Further comparison showed that the HYSA 0.2mM group was significantly higher than the HYSA 0.4mM group, the HYSA 0.8mM group and the HYSA 1.6mM group. So the 0.2mM HYSA concentration was chosen as the experimental condition. The cells treated with different concentrations of high ox-LDL showed statistically significant 24h survival rates compared with the control group as shown in Figure 1B. Two groups comparison showed that the survival rate of each group had a downward trend, high ox-LDL 5.0 μg/ml group> high ox-LDL10.0 μg/ml group> high ox-LDL20.0 μg/ml group> high ox -LDL 50.0 μg /ml group. Among them, the cell survival rate of the high ox-LDL 20.0 μg/ml group was 29.03%, which was lower than that of the control group. It was the best condition for constructing the cell injury model. Next, cells were treated with HYSA and high ox-LDL which had chosen to detect cell viability as shown in Figure 1C. The results showed that there was a statistically significant difference in cell viability between the different groups (p<0.05). Therefore, we finally determined the experimental concentrations of HYSA and high ox-LDL to be 0.2mM and 20.0μg/ml, respectively.

3.2. The effects of high ox-LDL and HYSA on cellular NO and LDH levels

Next, the LDH and NO contents in the cell culture medium were determined after treated with the HYSA, high ox-LDL or combination by lactate dehydrogenase colorimetric method and nitrate reduction method. The results of the analysis were shown in Figure 2A/B. The differences in NO and LDH contents between the four groups were statistically significant (p<0.05). Further comparison, the NO content (μM) showed high ox-LDL group < control group, high ox-LDL group < high ox-LDL + HYSA group. The LDH content (U/dL) was expressed in the control group < HYSA group < High ox-LDL + HYSA group < High ox-LDL group. Therefore, it was easily to see that high ox-LDL causes HCAECs damage to increase the release of LDH and decrease the release of NO. When HYSA was applied at the same time, the release of LDH was reduced and the release of NO was increased compared with the application of high ox-LDL alone. HYSA inhibited the damage of HCAECs by high ox-LDL and protects HCAEC.

3.3. Effects of HYSA on high oxLDL-induced cell apoptosis

To investigate the effect of HYSA on high oxLDL-induced HCAECs apoptosis, TUNEL analysis was used in this study. As shown in Figure 3, compared with the control group and the HYSA group, a large number of green fluorescent apoptotic cells were observed under the fluorescence microscope after treatment with high ox-LDL. However, the green signal was weakened and the apoptosis decreased after co-treatment with HYSA. Representative images and summary data for TUNEL were shown in Figure 3A/B. Compared with the control group, high ox-LDL increased the apoptotic rate from 2.02% to 46.16%. After co-treatment with HYSA, the apoptosis rate was significantly reduced to 31.88%. Therefore, it could be concluded that the treatment of HYSA inhibits high ox-LDL-induced apoptosis.

3.4. Effects of HYSA on oxLDL-induced LOX-1, eNOS mRNA and protein alteration

As shown in Figures 4 and 5. High ox-LDL treatment highly increased LOX-1 protein and mRNA expression and inhibited eNOS mRNA and protein expression. HYSA significantly attenuated oxLDL-induced up-regulation of LOX-1 and decreased eNOS expression. The experimental data indicated that HYSA inhibited the damage of HCAECs by high ox-LDL at the protein and gene level. Its specific regulatory mechanism was worthy of in-depth study, which was the focus of our next class.

4. Discussion

HYSA has been shown to have a broad spectrum of biological effects including, but not limited cardiovascular effects. Many studies have shown that HYSA has a significant anti-oxidative and neuroprotective effects[10,21,22] and also plays an active role in protecting acute liver, kidney, lung injury and reducing fibrosis and anti-tumor activity[23-29]. More significantly, some researchers have found that HYSA can inhibit the proliferation and migration of vascular smooth muscle cells and inhibit angiogenesis[30,31]. It is well known that an important mechanism of in-stent restenosis (ISR) after percutaneous coronary intervention (PCI) is the excessive proliferation of smooth muscle cells. The study found that HYSA could inhibit the damage of HCAECs induced by high ox-LDL and promoted the rapid repair. Here we proposed a hypothesis. A combination of HYSA promoted endothelial cell repair with rapamycin. Another one was paclitaxel inhibiting smooth muscle cell proliferation which could be used as a composite drug-eluting stent (DES) on the basis of
the existing scaffold to prevent ISR. The patient prompted rapidly re-endothelialization of blood vessels while inhibiting excessive proliferation of smooth muscle cells, reducing postoperative restenosis rate, shortening postoperative anticoagulation time in PCI patients, and reducing the incidence of postoperative complications and so on. It would be greatly benefit implications for both hospitals and society.

Ox-LDL formed by the oxidation of low-density lipoprotein (LDL) is a key event in the development of atherosclerosis[32]. With physiological conditions, LOX-1 expression levels are low, but under inflammation, oxidative stress and related stimuli, LOX-1 rapidly mobilizes and participates in the endothelial degradation process of ox-LDL, and the expression of LOX-1 gene and protein is up-regulated. Thereby can reduce the cellular lipid load[33]. Ox-LDL is the most potent activator of LOX-1, and ox-LDL dose- and time-dependently increases mRNA and protein levels of LOX-1[34,35]. This in turn increases LOX-1 mediated ox-LDL uptake, by accelerating the progression of atherosclerosis. LOX-1 causes a series of processes including endothelial dysfunction, activate of inflammatory factors, vascular smooth muscle cells (VSMC) migration and proliferation, platelet activation and foam cell formation, all of which promote atherosclerosis[36]. Further studies of LOX-1 indicate that the LOX-1 gene structural genetic polymorphism are associated with the risk of atherosclerotic cardiovascular events. LOX-1 is a transmembrane receptor, and its mediated mechanisms and signaling pathways have also been extensively studied. For example, the rapamycin drug-eluting stent commonly used in clinical practice inhibits the excessive proliferation of VSMC by inhibiting the mTOR/NF-κB pathway by preventing in-stent restenosis after PCI[37]. Therefore, LOX-1 is involved in the development and progression of atherosclerosis and its complications. LOX-1 has become a promising potential therapeutic target for atherosclerosis and related cardiovascular diseases.

Endothelial cell dysfunction is an early hallmark of atherosclerosis characterized by persistent inflammatory responses, oxidative stress and impaired production of NO [38]. This study found that in the high ox-LDL group, a large amount of cytoplasmic LDH was released. The cell damage was heavier, and the expression of LOX-1 was increased at the transcriptional and translational levels, which led HCAECs to be dysfunctional. However, the release of cytoplasmic LDH was reduced after co-treatment with HSYA. HSYA could reverse HCAECs damage caused by ox-LDL and protect endothelial cells to some extent. At the same time, the experiments further confirmed that HSYA increased the expression of eNOS at the transcriptional and translational levels, promoted the production of NO by HCAECs, and inhibited ox-LDL-mediated cell injury. In addition, LOX-1 was abundantly expressed during apoptosis, and the expression of mRNA and protein of eNOS were up-regulated during HSYA interference. The results indicated that HSYA also had protective effects on endothelial cells. In summary, it is not difficult to find that HSYA has a protective effect on HCAECs injury induced by high ox-LDL, which can reverse the damage of HCAECs caused by high ox-LDL, and it may play an immeasurable role in the treatment and prevention of atherosclerosis. It is also of great value in promoting rapid re-endothelialization after PCI and early prevention of ISR. This is the focus of the next study.

5. Conclusion

This study suggested that HSYA inhibited high ox-LDL-induced HCAECs injury and promoted HCAECs repair, which provided new and promising treatments for coronary heart disease. And HSYA could reverse the damage of HCAECs caused by high ox-LDL, might play an immeasurable role in the treatment and prevention of atherosclerosis.

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